



Saskatchewan
Ministry of
Agriculture



RESEARCH

ADF

AGRICULTURE

DEVELOPMENT

FUND

20050729

**STUDY OF STARCH GRANULE SIZE DISTRIBUTION
TO IMPROVE BARLEY GRAIN QUALITY**

Funded by: The Agriculture Development Fund

March 2010

Prepared by: University of Saskatchewan (U of S)

FINAL REPORT



Final Report

ADF Project # 20050729

STUDY OF STARCH GRANULE SIZE DISTRIBUTION TO IMPROVE BARLEY GRAIN QUALITY

FINAL REPORT - May 2006- January 2010

Principal Investigator: Dr. Ravindra Chibbar

Co-investigators: Dr. Monica Bâga & Dr. Brian Rossnagel

**Department of Plant Sciences and Crop Development Centre,
College of Agriculture and Bioresources, University of Saskatchewan
Saskatoon, Saskatchewan**

Table of contents

Title	Pg
Executive summary.....	2
Introduction and Objectives.....	3
Materials and Methods.....	6
Results.....	10
Objective 1.....	10
Phenotypic confirmation of high A-type starch granule content in two barley lines (Finnish and Japanese) grown under different Saskatchewan agro-climatic conditions	
Objective 2.....	11
Introgress high A-type starch granule trait in Saskatchewan barley cultivars	
Objective 3.....	21
Elucidate the genetic and molecular mechanism underlying starch granule development in barley	
Objective 4.....	33
Develop targeted molecular markers for starch granule size distribution based on molecular analyses of natural variants and use a doubled haploid barley population (Steptoe x Morex or Harrington x TR 306) to identify novel QTL associated with starch granule development in barley.	
Conclusions and Recommendations.....	35
Acknowledgements.....	36
References.....	37
Other.....	40

ABSTRACT/ SUMMARY

The main objective of this project was to develop barley lines with altered granule size distribution, specifically reduced amount of B-type starch granules. The current barley cultivars endosperm starch is made of 10-15 vol-% of B-type granules and the remainder is A-type granules. B-type starch granules clog the filters during the mashing process and yield less extract as compared to large A-type granules, therefore the malting barley industry desires barley cultivars with reduced B-type starch granules. Screening of barley germplasm for altered granule size distribution identified a Japanese barley line with reduced small size (B-type) starch granules. The results of field trial (2006) at three locations confirmed the selected Japanese line produces a higher than normal percentage of A-type starch granules. The large A-type starch granules of control lines had higher amylose concentration as compared to small B-type starch granules. The identified Japanese line showed no difference in amylose concentration between the large A-type and small B-type starch granules. Similar type of starch amylose concentration suggested that identified barley line produced uni-modal (A-type) type of starch granules.

The second objective of this project was to introgress the unimodal or reduced small size starch granule trait in to Canadian cultivars. The identified Japanese line was backcrossed with three Canadian varieties (CDC Kendall, CDC Clyde and CDC Copeland). All the three crosses resulted in barley lines with reduced B-type starch granules. Starch granule size analysis of a cross between CDC Kendall and the Japanese line revealed the development of a line with a unimodal type starch granule distribution. Microscopic image analysis showed the presence of a large number of very small granules, but their contribution to total volume percentage was insignificant. We hypothesize that these very small starch granules are indeed large granules whose development has been arrested. To prove this hypothesis different physical, biochemical and molecular techniques were used.

Biochemical and molecular analysis revealed that amylose concentration is associated with starch granule size. Exploiting the synteny within grass genomes we identified a QTL on wheat genome associated with total amylose content and starch swelling power, two traits associated with starch granule size. A novel form of starch synthase III associated with the identified QTL was characterized by DNA sequencing.

Detailed morphological, biochemical and genetic analysis was done on F₅ bulk (88 lines) and F₅ single seed descent (66 lines) lines derived from a cross between CDC Kendall and the identified Japanese line. Determination of starch granule size distribution using a Malvern Mastersizer-2000 laser-diffraction analyzer, revealed 20 lines with reduced volume of small starch granule (<5 microns). Some of these lines do not have starch granules less than five microns. SDS-PAGE analysis of starch granule proteins revealed that the Japanese line showed two additional polypeptides with molecular mass of 66 and 53 kD. These polypeptides did not cross react with antibodies recognizing starch synthases, branching enzymes or de-branching enzymes. Analysis of starch biosynthetic enzyme in developing endosperm revealed similarity in the phosphorylating and amylolytic isozyme.

In conclusion, barley lines with reduced B-type or only A-type starch granules were developed. Biochemical and genetic studies have resulted in some markers, which need to be confirmed by detailed biochemical and molecular analysis using the genetical material developed in this project. These genetic markers will expedite the development of new barley cultivars with increased A-type starch granules to meet the need of barley industry.

INTRODUCTION

Starch is the major and the most abundant storage polysaccharide in seed, fruits, tubers and roots of higher plants. It occupies the status of second largest biomass next to cellulose on earth. It acts as a source of energy during dormancy and developmental stages. Cereal and tuber starch are staple food in the human diet. Almost 50 percent of the calories in human diet are supplied by starch based products.

Starch is stored as discrete water-insoluble granules with an internal lamellar structure. The starch granule contains two distinct polysaccharide types, amylose and amylopectins. A normal starch granule is made of one quarter amylose and three quarters amylopectin. Amylose is essentially a linear polymer of glucose residues with α -(1-4) linkages, that is sparsely branched with α -(1, 6) bond (one in 1000 glucose residues) (Takeda and Hizuluri 1987), whereas amylopectin is a large branched molecule composed numerous short α -1, 4-linked glucose residues with α -(1,6) branch points after every 24 to 30 glucose residues on average.

The ratio of amylose to amylopectin is an important factor determining starch properties and its end use. Mutants which have as much as 70% amylose have been reported in different cereal crops. In contrast, waxy mutants which have undetectable amylose concentration also occur in nature or have been genetically synthesized.

Amylose

Initially amylose was considered α -(1-4) linked linear molecule but is now well established that it contains few α -(1-6) branches too. The number of branches in amylose in turn varies with molecular size and botanical source (Hizkuri et al.1981, Takeda et al. 1984). In case of cereals molecular size of amylose is smaller than tubers (Takeda and Hizukuri 1987; Jane and Chen 1992).

Amylopectin

Amylopectin which occupies ~75% part of starch granule is a branched polysaccharide composed of several short (1,4)- α -glucan chains, interlinked by (1,6)- α -linkages. A small part ~5% of theses residues has both types of linkages. It is much larger than amylose. The huge structure makes it much more complex than amylose. It is responsible for the crystalline nature of the starch granules. Native starch shows about 15-45% crystallinity (Zobel 1988). HPEC-MALLS studies on amylopectin from different botanical sources showed weight-average molecular weight ranging from 10^8 to 10^9 Da (Fishman et al 1996, Yoo and Jane 2002). Amylopectin of waxy starch was found to be heavier than normal or high amylose lines (Yoo and Jane, 2002). According to Jane 2006 this happens due to the partitioning of ADP glucose as substrate for amylose and amylopectin synthesis in normal and high amylose lines while in case of waxy lines it is solely used for amylopectin synthesis. Length of amylopectin branch chain is longer in small granules than bigger granules (Jane and Shen 1993; Pan and Jane 2000)

Granule size distribution and malting quality in barley

Starch is generally stored in plant's amyloplast in the form of granules which could either be of uniform size (uni-modal) or two different sizes (bimodal).

Four members of triticeae family, wheat, barley, rye and triticale are unique in having bimodal size distribution in endosperm starch granules. Based on the diameter, these granules can be grouped as large A-type lenticular ($>10\mu\text{m}$) and small B-type oval shaped granules ($<10\mu\text{m}$). Development of A granules occur 4-6 days post anthesis while B granules develop later, 12-14 days post anthesis (Fig I)

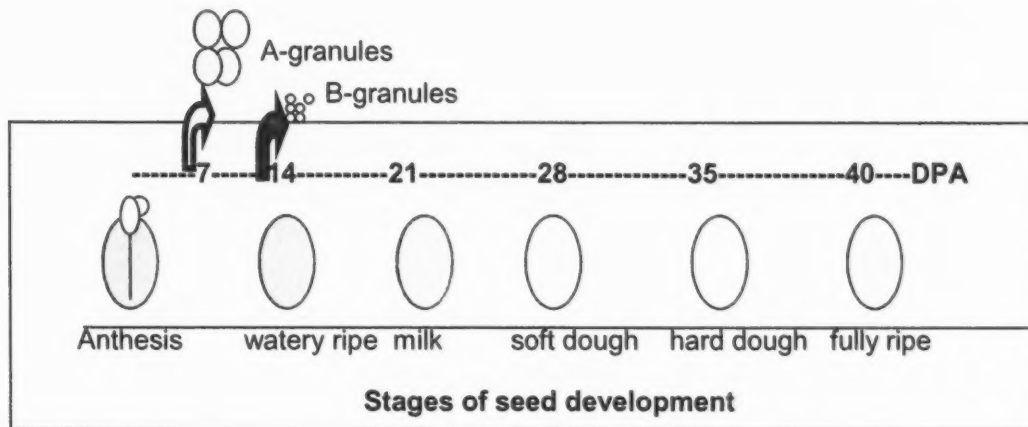


Fig I. Diagrammatic representation of stages of seed development and A and B granule synthesis in cereals like wheat and barley

In a mature cereal grain large A type granules occupy only 10-15% part of the grain by number but more than 80% by volume and account for most of the starch by weight (~90%). A and B type granules differ slightly in their amylose content, the overall lipid composition, and the amount of proteins attached to the surface (Morrison 1995; Meredith et al. 1981). A-type starch granules are more susceptible to hydrolysis by α -amylase II than B granules at 65°C (MacGregor and Ballance, 1980) the temperature under which mashing occurs during brewing. During the mashing process in brewing, hydrolytic enzymes are more active on the large granules as compared to small granules (Borem et al. 1999), and therefore, A-type starch granules contribute more to malting quality than B-type granules. Small starch granules may also cause problems during malting as they adhere easily to proteins leading to clogging of filters. Thus, there has been a desire from the malting industries to produce malt from barley lines with a lower content of small starch granules (Pierce 1991).

In comparison to other crops malting barley undergoes more quality constraints and is one of the few crops sold consistently on cultivar basis. The requirement of desired cultivar is specified by commercial malsters and brewers to maintain high quality product. This project focus on the development of barley lines with reduced B granule content and study the biochemical and molecular mechanisms controlling the process of starch granule development.

Objectives

The long term objective of this project was to develop barley line with reduced B granule content desired by the malting industry. The project was initiated with four main research objectives

1. Phenotypic confirmation of high A-type starch granule content in two barley lines (Finnish and Japanese) grown under different Saskatchewan agro-climatic conditions
2. Introgress high A-type starch granule trait in Saskatchewan barley cultivars
3. Elucidate the genetic and molecular mechanism underlying starch granule development in barley
4. Develop targeted molecular markers for starch granule size distribution based on molecular analyses of natural variants and use a doubled haploid barley population (Steptoe x Morex or Harrington x TR 306) to identify novel QTL associated with starch granule development in barley

The results will be presented and discussed under each objective. Methodology used in meeting these objectives will be discussed in materials and methods section.

MATERIAL AND METHODS

Plant material and growth conditions

Initial screening for low B granule content in barley (*Hordeum vulgare* L.) germplasm resulted in the identification of a Finnish and a Japanese line with low B granule content. To confirm the phenotype a six entry barley trial (year 2006) was planted at three different sites, Preston, Kernen and Seed Farm at the University of Saskatchewan, Saskatoon with three control cultivars, CDC Battleford, CDC Dolly and Harrington. Finnish barley line was rejected due to inconsistency of the results in field and green house conditions. The Japanese line, which showed consistently lower concentration of B-type starch granule, was crossed with three barley varieties: CDC Clyde (six-row malting variety), CDC Copeland and CDC Kendall (two-row malting cultivars). In addition, recombinant inbred lines (RIL) were also developed from a cross between the Japanese line and CDC Kendall. Seeds of these RIL's for F5 population were grown in the month of May, 2008 in Seed farm, University of Saskatchewan. Single seed descent of this population were grown at green house, University of Saskatchewan with 25/20 °C day/night temperatures, 570–620 mmol/m²/s light intensity and 18 hour photoperiod.

Starch extraction:

Starch was extracted from barley flour using modified Demeke et al., (1997) procedure. Hundred milligrams flour from each sample was weighed into a microfuge tube (2 ml) and 1.5ml water was added. The slurry was centrifuged at 15,000 g for 10 min. The pellet was re-dissolved in 200 µl water, vortexed and layered on 1.5 ml, 70% (w/v) CsCl. This was centrifuged at 13,000 g for 30 min to pellet the starch granules. This crude starch granules pellet was washed three times with 1 ml wash buffer (55 mmol L⁻¹ Tris-HCl, pH 6.8, 23 g L⁻¹ sodium dodecyl sulfate (SDS), 100 mL L⁻¹ glycerol) containing β-mercaptoethanol (19:1 v/v). This process helps in cleaning the proteins adhered to starch granules. External protein free starch granules were then washed two times with 1 ml distilled water. All the washing steps were carried in microfuge at 4,500 g. For lipid removal the washed starch pellet was treated with 1 ml acetone and centrifuged at 4,500 g for 10 min. The purified starch pellet was dried over silica gel in desiccators for 48 hours.

Modified starch extraction for granule size determination using master sizer analyser:

Coarsely ground barley grains (4-6) were steeped in 0.02N HCl (2ml) overnight at 4°C. After neutralization with 0.2 N NaOH and centrifugation at 4000 g, the residue was crushed in 2 ml of 0.1 M Tris-HCl buffer containing 0.5% NaHSO₃ (pH 7.0), followed by enzyme cocktail (proteinase 15 U/g barley, lichenase 2 U/g barley and β-xylanase(8 U/g barley) (You and Izydorczyk 2002). The samples were treated overnight and filtered through 100 µm nylon filter. The crude starch slurry was centrifuged for 10 min at 4000 g. The precipitate was re-suspended with 200 µl of water, layered over 1 ml of 80% (w/v) CsCl solution and centrifuged at 13,000 g for 30 min. The starch pellet was washed twice with water and then acetone. It was allowed to air-dry overnight.

Granule size distribution:

Starch (40 mg) was suspended in 1 ml water and dispersed in sample holder attached to laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments, Malvern, England). For analysis in Malvern Mastersizer-2000 refractive indices of 1.31 for water and 1.52 for starch

were used as standard. 1700 rpm was used as dispersant speed and sonication was allowed during analysis of the sample. Each sample was analysed two times and average of two was recorded. The volume percentage of small B-type starch granules was determined using a cut-off diameter of 5.0 μm .

Separation of A and B granules

The extracted starch was separated into A and B granules using modified Percoll method (Peng et al. 2000). A starch sample (200mg) was suspended in 200 μl of distilled water and layered over 1ml of 70% percoll in a microfuge tube (2 ml). It was centrifuged at 100 g for 1 min and supernatant was collected in 15 ml tube labeled B-granule. The process was repeated three times with the remaining starch pellet. The same process was done three times with 100% percoll and supernatant was collected in the same B-granule labeled tube. The final pellet was treated as 'A granule' and washed two times with water and then acetone by vortexing and centrifuging at 11,000 g for 10 min. The collected B granule fraction was centrifuged at 11,000 g for 20min and the resultant B granule pellet was washed with water and followed by acetone. A and B granule pellets were allowed to air dry overnight.

Total starch concentration

Total starch concentration was determined according to the Megazyme method (Megazyme International Ireland Ltd, Wicklow Ireland) (McLeary et al. 1994). Briefly, a 100 mg sample was suspended in 0.2 mL of 800 mL L^{-1} ethanol to aid dispersion and mixed vigorously using a vortex mixer. The sample was incubated at 100 °C for 8 min and mixed every 2 min with 300 U of α -amylase (AA) in 50 mmol L^{-1} 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (pH 7) to hydrolyse starch to dextrans. Subsequent incubation with 4 mL of 20 mmol L^{-1} sodium acetate buffer (pH 4.5) and 0.1 mL of 20 U amyloglucosidase (AMG) at 50 °C for 30 min hydrolysed dextrans to glucose. The reaction mixture was diluted to 100 mL and a 1 mL aliquot was added to 3 mL of glucose determination reagent and incubated at 50 °C for 20 min. Total starch was calculated as free glucose by measuring the absorbance at 510 nm and using the formula:

$$\begin{aligned}\text{Starch \%} &= A_A \times F \times \frac{FV}{0.1} \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180} \\ &= A_A \times \frac{F}{W} \times FV \times 0.9\end{aligned}$$

where:

A_A = Absorbance (reaction) read against the reagent blank.

F = $\frac{100 \text{ (}\mu\text{g of D-glucose)}}{\text{absorbance for } 100 \mu\text{g of glucose}}$ (conversion from absorbance to μg)

FV = Final volume i.e. equals 100 ml or 10 ml in examples (a) and (b); 100 or 10.4 ml in example (c); 100 ml or 10.0 ml in example (d); and 100 ml or 10 ml in example (e).

0.1 = volume of sample analysed

$\frac{1}{1000}$ = Conversion from μg to mg .

$\frac{100}{W}$ = Factor to express 'starch' as a percentage of flour weight.

W = The weight in milligrams ('as is' basis) of the flour analysed.

$\frac{162}{180}$ = Adjustment from free D-glucose to anhydro D-glucose (as occurs in starch).

Starch % w/w (dry wt. basis):

$$= \text{Starch \% w/w (as is)} \times \frac{100}{100 - \text{moisture content (\% w/w)}}$$

Amylose determination

A high-performance size exclusion chromatography (HP-SEC) method was used to determine amylose concentration (Demeke et al. 1999). A 5 mg starch sample was suspended in 5 mL of distilled water in a glass tube and incubated at 130 °C for 30 min. To 1 mL of this gelatinised starch solution, 55 µL of 1 mol L⁻¹ sodium acetate (pH 4) was added with vigorous mixing, followed by 4 U of isoamylase to debranch the starch. After 4 h of incubation at 40 °C the debranching reaction was stopped by boiling for 20 min to inactivate isoamylase. The debranched starch solution was freeze-dried. The freeze-dried sample was dissolved in 200 µL of 990 mL L⁻¹ dimethyl sulfoxide (DMSO) and centrifuged in a microfuge at 15,000 g. A 40 µL aliquot of the supernatant was injected into a PLgel MiniMix-C guard column attached to a PLgel MiniMix 4.6 mm i.d. column (Polymer Laboratories, Inc., Amherst, MA, USA) to separate amylose and amylopectin using a high-performance liquid chromatography (HPLC) system comprising a Waters 600 controller, Waters 610 fluid unit, Waters 717 Plus autosampler and Waters 410 differential refractometer (Waters Corporation, Milford, MA, USA). The data were collected and analysed using Empower software (Waters Corporation). Starch samples, column and detector were maintained at 40, 100 and 45 °C respectively. The eluent used was 990 mL L⁻¹ DMSO at a flow rate of 0.2 mL min⁻¹. The amylose concentration was calculated by integration of the peak area corresponding to amylose with respect to the peak area corresponding to both amylose and amylopectin.

Starch Granule Bound Proteins Extraction

Five milligram (5mg) starch samples were suspended in 150µl SDS-PAGE sample buffer with β-mercaptoethanol (4:1, v/v), vortexed for 1min and heated for 5min in a boiling water bath. Samples were cooled immediately on ice for 5 min and 15min for normal/high amylose lines and waxy lines respectively, and centrifuged at 15,700 g for 15min. Equal volume of protein (40µl) from each sample was loaded on a SDS-PAGE gel and separated by gel electrophoresis.

Separation of Starch Granule Bound Proteins by (SDS-PAGE) Electrophoresis:

Starch Synthase proteins (SSII isoproteins) and Granule Bound Starch Synthase I (GBSSI) were separated on a discontinuous SDS-PAGE with 10% and 20% resolving gels respectively and 5% stacking gels for each as previously described by Demeke et al. (1997). For each gel type 40 ml of the resolving gel was poured into Biorad electrophoretic gel apparatus and allowed to polymerize (within 1hour) before the stacking gel was layered on the resolving gel. A 1.5 mm, 15 well comb was inserted and 40 µl denatured protein sample from each barley line was loaded into respective well. The protein standard from Invitrogen (High range Bench Mark, 10747-012) was prepared by adding 1.1 µL protein standard to 28.9 µL of starch sample buffer and boiling in water bath for 3 min. Gel was run on a vertical electrophoretic apparatus (Biorad) in 1x running buffer with 0.1% (v/v) β-mercaptoethanol at 15 mA/gel.

Silver Staining of SDS-PAGE gel:

Starch Granule-bound polypeptides separated on SDS-PAGE were visualized by silver staining as described (Båga et al., 2000). The stacking gel was removed and gel was washed in Prefixative A (40% methanol v/v and 50% glacial acetic acid v/v) for 30 min and then in Prefixative B (5.45% methanol v/v and 7.6% glacial acetic acid v/v) for 30 min. In three

exchanges of 10 min each, the gel was washed in doubled distilled water (DD H₂O), followed by 30 min in Dithiothreitol (DTT) solution (3.3mg/mL, w/v). Gel was agitated for another 30 min in 300 ml of 0.1% (w/v) Silver nitrate solution, followed by washing briefly (10 sec) with DD H₂O. Protein bands were developed by agitating gel in 150 ml developer (3% (w/v) Na₂CO₃ + 10% (v/v) Formaldehyde) twice for 30 sec each. At the third exchange, gel was agitated until desired band intensity was achieved and photographed.

Immuno-detection analysis

Immunoblotting analyses of starch granule proteins were done following procedures described by Gao and Chibbar (2000). Starch granule proteins separated on SDS-PAGE were transferred onto nitrocellulose transfer membrane (Protran^R, Whatman) after equilibrating gels in transfer buffer for 30 min. Protein on membrane were blocked with 5% carnation milk in 1x Phosphate Buffered Saline (PBS) and incubated with respective antiserum against wheat starch granule proteins at 1:10,000 dilutions. The antigen-antibody complex was detected with goat anti-rabbit alkaline phosphate conjugate using BCIP/NBT immunoscreening color development kit (BIO-RAD, Hercules, CA.).

Phosphorylase and amylolytic enzyme activity

For analysis of Native PAGE/activity staining of phosphorylase and amylolytic enzyme immature barley grain at late milky stage were removed by the hull, pericarp and embryo was homogenized in ice cold pestle and mortar in buffer solution, which consisted of 50mM Hepes-NaOH (pH 7.4), 2mM MgCl₂, 50 mM 2 mercaptoethanol and 12.5% (v/v) glycerol. The homogenate was centrifuged at 11,000 g for 15 min. The supernatant was used as crude enzyme extract.

Native PAGE/activity staining of phosphorylase and amylolytic enzymes

Phosphorylase activity

Native PAGE was performed on 6% (w/v resolving gel containing 0.4% w/v glycogen) and 3.3% (w/v stacking gel) acrylamide by a little modification of Davis (1964) method. Electrophoresis was carried out at 4 °C at a constant current of 10 mA for 12 h. After electrophoresis the gel was rinsed with 20 mL of a solution of 50 mM HEPES-NaOH buffer (pH 7.4) and 20% (v/v) glycerol for 5 min on ice. The gel for detection of phosphorylase was incubated for 30 min at 30°C in 20 mL of the reaction mixture, which consisted of 50 mM HEPES-NaOH buffer (pH 7.4), 50 mM Glc-1-P, 2.5 mM AMP and 10% (v/v) glycerol. The gels were placed in an iodine solution [0.1% (w/v) I₂ and 1% (w/v) KI] (Nishi et al. 2001).

Amylolytic activity

Native PAGE was performed on 7% (w/v resolving gel containing) and 5% (w/v stacking gel) acrylamide by a little modification of Davis (1964) method. Electrophoresis was carried out at 4 °C at a constant current of 10mA for 12 h. After electrophoresis the gel was incubated in 2% starch solution for 30 min. After incubation the gels were placed in an iodine solution that consisted of 0.1% (w/v) I₂ and 1% (w/v) KI.

RESULTS

Objective 1.

Phenotypic confirmation of high A-type starch granule content in two barley lines (Finnish and Japanese) grown under different Saskatchewan agro-climatic conditions. (2006-2007)

Our previous studies of barley lines grown in the greenhouse had indicated that two lines, a Japanese and a Finnish line, produce starch with higher A-granule concentration as compared to normal barley. These two lines along with three checks were selected for evaluation in the field. A six entry barley trial was planted at three different sites, Preston, Kernen and Seed Farm at the University of Saskatchewan, Saskatoon. The grains were harvested and starch was isolated from all the replicates. The results showed that the Japanese line had a consistently low B-type starch granule content as compared to the three control cultivars, CDC Battleford, CDC Dolly and Harrington. The Finnish line represented by two accessions, which had earlier indicated low B-type starch granule content in greenhouse experiments, showed normal percentage B granules in the field experiments. Thus, we do not believe the Finnish line differs significantly from normal barley lines with regard to B granule content (Table 1). The reason for variation in A/B granule ratios in starch produced in the green house and the field for the Finnish line is not clear, but environmental factors are known to influence starch granule development. The main conclusion from this milestone is that the Japanese line has produced consistently higher concentration of large A-type starch granules in the greenhouse, as well as, in all three field trials conducted (Table 1). Therefore, we have selected this line for further evaluation and have made crosses with Saskatchewan cultivars to increase the A-type starch granules as described in milestone 2. This completes the work under milestone 1

Table 1. B granule content in grain of field-grown barley lines.

Barley lines	Site			average
	Kernen Farm	Preston	Seed farm	
CDC Battleford	80.9	88.6	77.5	82.3 $\pm$ 8.6
CDC Dolly	86.7	90.6	81.2	86.2 $\pm$ 6.2
Harrington	78.8	82.3	84.4	81.8 $\pm$ 4.5
Finnish 1	95.7	89.9	85.1	90.1 $\pm$ 5.1
Finnish 2	81.8	75.9	82.4	80.0 $\pm$ 6.4
Japanese	60.2	67.5	65.3	64.3 $\pm$ 3.1

Objective 2

Introgress high A-type starch granule trait in Saskatchewan barley cultivars (1/11/2006 – 31/3/2009).

The Japanese line, which showed consistently higher concentration of large A-type starch granules was crossed with three barley varieties: CDC Clyde (six-row malting variety), CDC Copeland and CDC Kendall (two-row malting cultivars). In addition, recombinant inbred lines (RIL) were also developed from a cross between the Japanese line and CDC Kendall.

As a first step towards analyzing a large number of samples, we decided to use a Malvern Mastersizer instead of microscope image analysis system. Microscope image analysis system was used to confirm representative results obtained with Malvern Mastersizer. To use a Malvern Mastersizer, the starch powder has to be prepared in a manner that the starch granules are completely separated. We had to modify the method for starch isolation. After trying several methods, we developed a modified method (for small grain sample) based on a method by You and Izydorczyk (2002). Details of the method are given in Material and Methods section.

Breeding lines (F₂) developed from this cross showed clear segregation for granule size distribution trait. The results show that the population was segregating for the trait and a line with majority A-type granules was discovered. To confirm it further F-5 bulk (88 lines) and F-5 single seed descent population (66 lines) was checked for distribution for granule size. Two independent experiments with starch extracted from middle region of single head and starch extracted from mixture of seeds from different heads for a line were analyzed for starch granule size distribution using a Malvern Master Sizer. For analysis in Malvern Mastersizer-2000 laser-diffraction analyzer (Malvern Instruments Ltd, Malvern, UK) refractive indices of 1.31 for water and 1.52 for starch were used as standard. 1700 rpm was used as dispersant speed and sonication was allowed during analysis of the sample. 40 mg of well dried starch suspended in 1 ml water was used for analysis. Each sample was analysed two times and average of two was recorded. Laser obscuration was kept between 12-14% during sample addition, as recommended by the manufacturers.

CDC Kendall contains 11-13% B granule content while in case of Japanese line it ranged from 3-6%. The graph of Japanese line was skewed more towards medium size granules. (Fig. 1) Some of the selected breeding lines had unimodal kind of distribution. The screening of F-5 (bulk), F-5 (single seed descent) population showed clear segregation for granule size distribution trait (Fig. 2). Six lines from F-5 (bulk), and fourteen from F-5 single seed descent were selected for repeat trial in field. All the lines of F-5 (bulk), F-5 single seed descent were grown in a green house. Phenotypic data for morphological characters like plant height, awn length, presence/absence of hull and flowering time was analysed for the green house trial.

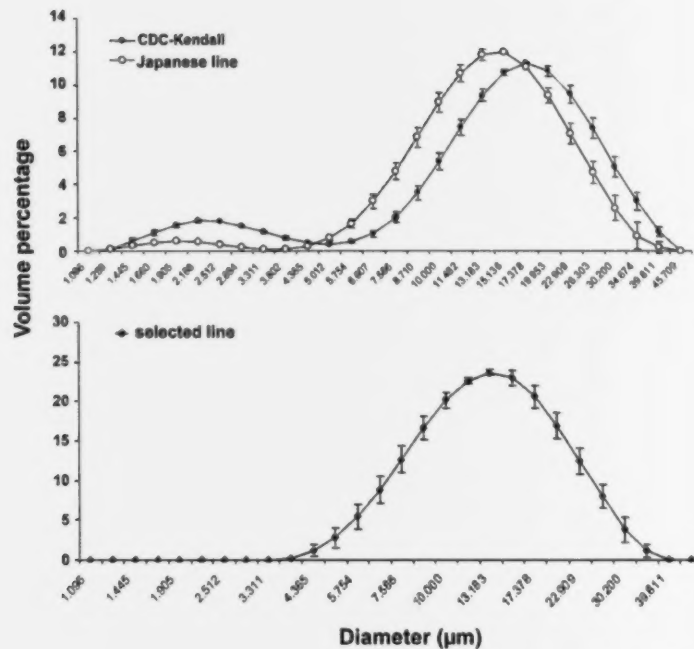


Fig. 1. Granule size distribution (volume based) in parents (CDC-Kendall and Japanese line) and a selected breeding line. Error bars represent the standard deviation for the volume percentage value in three independent trials.

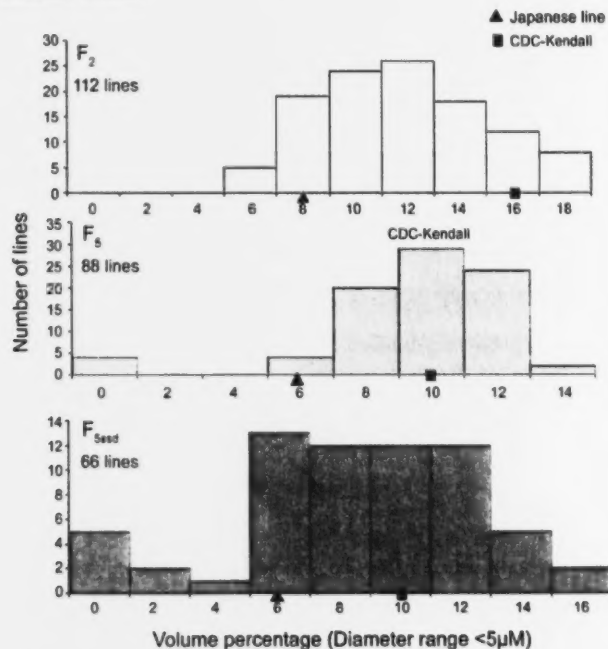


Fig. 2. Segregation of starch granule size in F_2 , F_5 (bulk), F_5 single seed descent population derived from CDC-Kendall $\times$ KK-2 for volume percent of $<5 \mu\text{m}$ diameter granules. $\blacktriangle$, $\blacksquare$ indicate position of parental lines KK-2 and CDC Kendall respectively.

Amylose content in the parent and selected breeding lines

Amylose content determined by HPSEC method varied in selected lines from 23-27%. The Canadian and Japanese line showed ~25 and 27% amylose content respectively. The selected breeding lines appears to follow no clear trend towards increasing or decreasing side (Fig. 3)

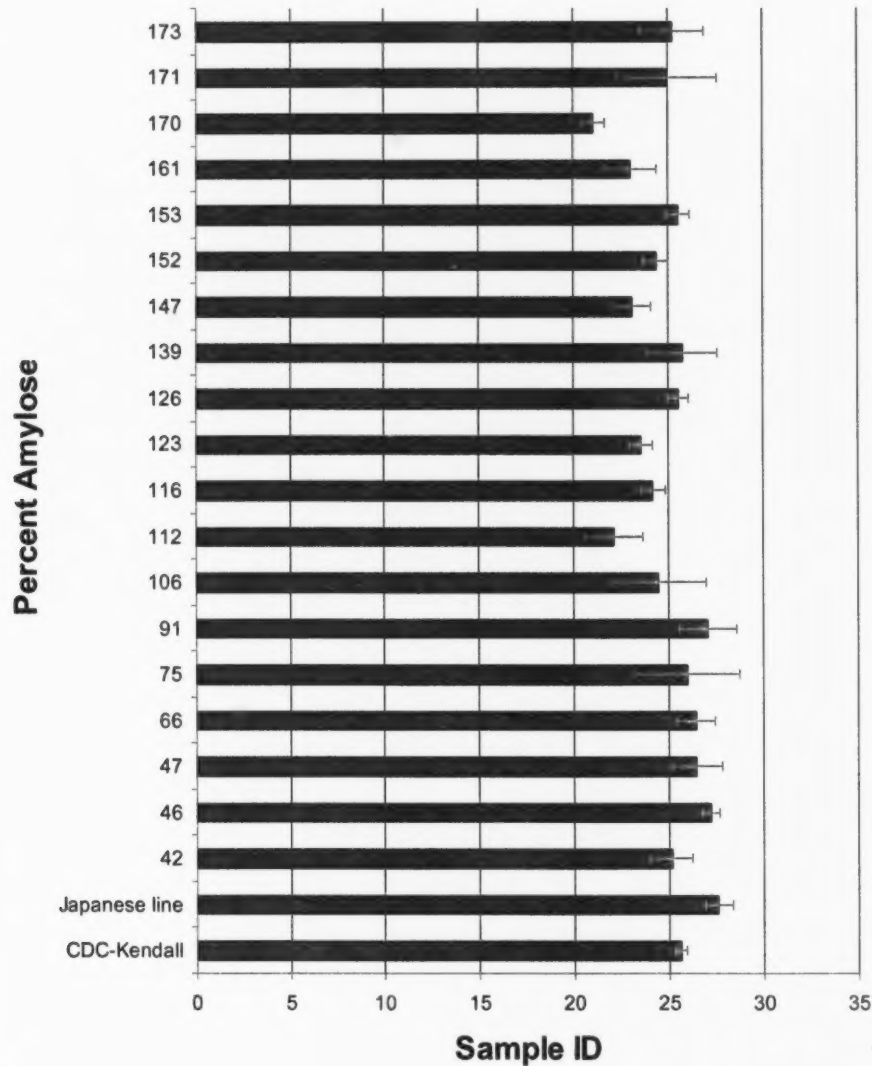


Fig. 3. Percent Amylose content determined by HPSEC method in parents (CDC-Kendall and Japanese line) and selected breeding lines. Error bars represent the standard deviation for the amylose concentration value in three independent trials

Amylose content in the separated A and B granules of parent and selected breeding lines

Amylose content in the A and B granule fraction of four out of eight selected breeding lines from F-5 bulk population was approximately similar (Table 2). This further strengthens our hypothesis that the smaller granules of selected breeding lines and Japanese parent are actually large granules which somehow failed to grow in size. The line coded 66 though selected, could not be analysed due to insufficient grains.

Table 2. Percent Amylose content determined by HPSEC method in A and B granule fraction of parents (CDC-Kendall and Japanese line) and selected breeding lines (F5) in three independent trials

Sample ID	A-granules	B-granules
CDC-Kendall	27.31 $\pm$ 0.28	24.51 $\pm$ 0.50
KK-2	30.98 $\pm$ 0.15	29.11 $\pm$ 0.67
25	29.44 $\pm$ 0.76	28.18 $\pm$ 1.47
26	29.65 $\pm$ 0.23	27.53 $\pm$ 0.35
42	26.72 $\pm$ 0.98	24.81 $\pm$ 0.87
43	29.37 $\pm$ 0.51	21.51 $\pm$ 1.67
46	30.64 $\pm$ 1.77	27.75 $\pm$ 1.45
47	29.68 $\pm$ 1.22	28.89 $\pm$ 1.02
66	NA	NA
75	31.54 $\pm$ 2.49	26.55 $\pm$ 0.91

Segregation of morphological characters in breeding lines derived from CDC Kendall x Japanese line (green house trial)

In an attempt to correlate granule size distribution to morphological character associated specifically for Japanese line green house trial of this cross was conducted and data was recorded for different morphological characters

Japanese line selected for these crosses in addition to granule size differs from Canadian barley lines in several morphological characters including

- Plant Height
- Number of rows in head
- Awn length
- Presence of hull +/-
- Angle of flag leaf

- Distance between head and flag leaf
- Length and width of foliar leaf
- Number of tillers
- Spring/winter growth habit

The selected Japanese parent line is semidwarf approximately half (36 cm) in height as compare to CDC Kendall (~60 cm). Awn length in case of CDC Kendall is 17 cm while average awn length for Japanese line is 2.7cm. The breeding lines obtained from this cross were also segregating for these characters (Fig. 4.).

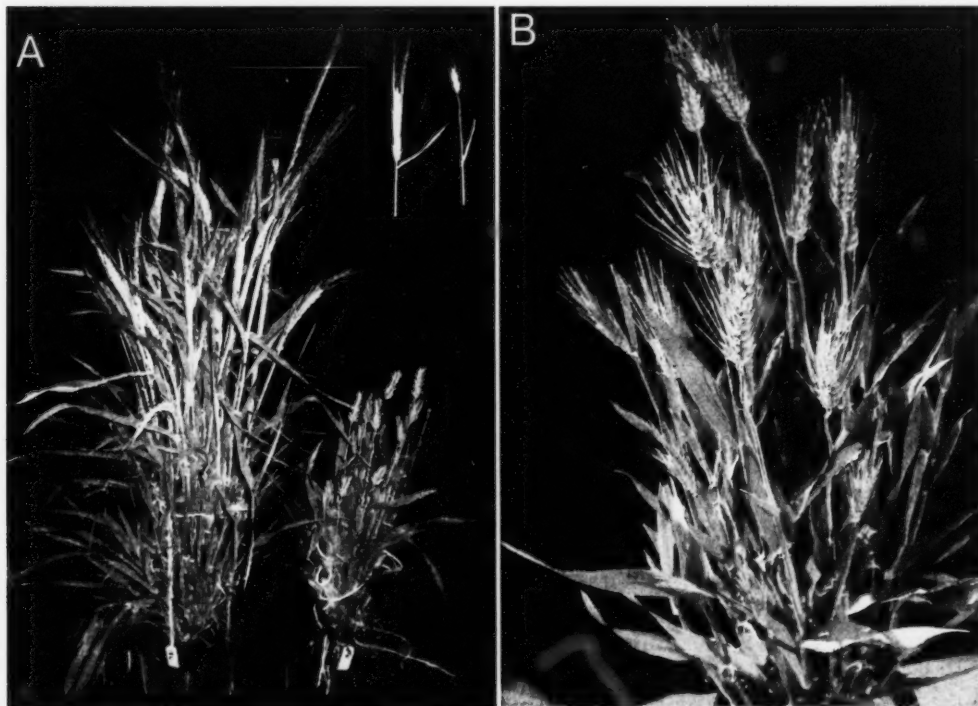


Fig. 4. CDC Kendall and Japanese parent (A); inset represent enlarged view of the parental spikes; The Japanese semidwarf barley line with six row spike and short awns (B).

Some of the breeding lines screened in this study appeared morphologically similar to the Japanese parent but differed in granule size distribution.

The Japanese showed significant variation in the plant height in the two sets of samples analysed for this study (Fig. 5).

None of the morphological features taken in this study correlated with granule size distribution trait.

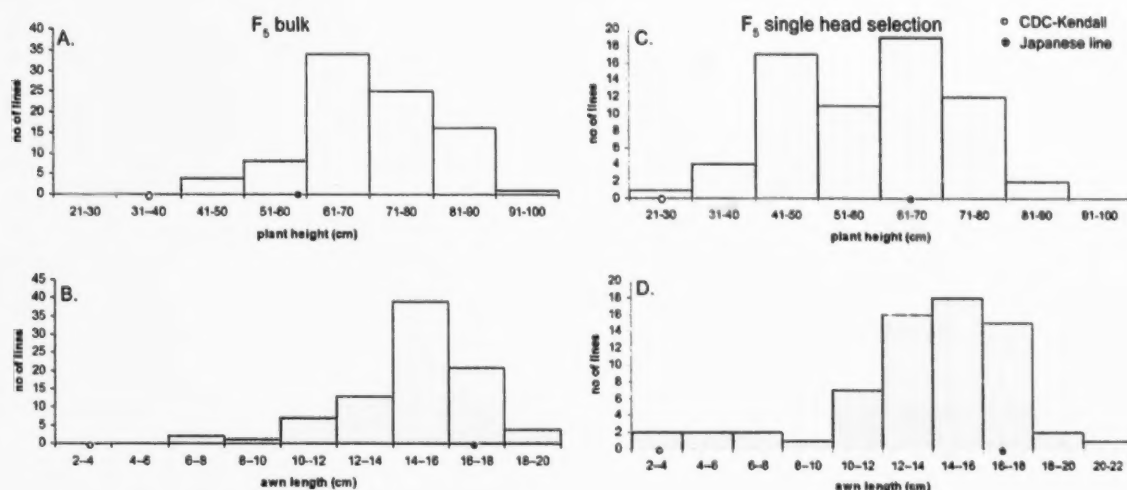


Fig. 5. Segregation of plant height and awn length in F_5 (bulk) A,B and F_5 (single head selection) C, D breeding lines derived from CDC-Kendall x KK-2. Plants were grown in green house and data was recorded at the harvest time.

The Canadian barley genotype CDC-Kendall has spring growth habit while the Japanese KK-2 has winter growth habit. To assess the correlation between growth habit and altered starch granule distribution DNA marker based diagnosis was conducted on breeding lines developed from cross between CDC-Kendall and KK-2.

PCR-Based Markers Diagnostic for Spring and Winter Seasonal Growth Habit CDC Kendall x Japanese line

A DNA marker method given by Cockram et al (2009) was used determine spring and winter habit segregation in the breeding lines obtained from this cross. Several workers reported strong correlation between spring alleles and the presence of insertion-deletions (InDels) within VRNH1intron 1 (Fu et al., 2005; von Zitzewitz et al., 2005; Cockram et al., 2007ab, 2008; Szucs et al., 2007). Multiplex PCR devised by this group diagnose these InDels and deletion assayed by this correlate with observance of spring growth habit.

Primers

primer pair 1

HvBM5A-intron1-F3b (5'- CTTGCATGTGTTGTCGGTCT-3') and *HvBM5A*-intron1-R3b (5'- GCTGGGACAAGACTCTACGG-3') (expected product size 830bp)

primer pair 2 (*HvBM5A*-intron1-F1,

5'-GTTCTCCACCGAGTCATGGT-3'; *HvBM5A*-TE-R1,

5'-AGAGATGGAGGCATGGAGCA-3') amplifying a 488-bp product

primer pair 3

(*HvBM5A*-exon2-F1, 5'-TCCCAAGAAAAGTTGAACAACACCAG-3'; *HvBM5A*-exon2-R1, 5'-ATTAGGTTACATCATTCGACCA-3') (expected product size 616/574-bp)

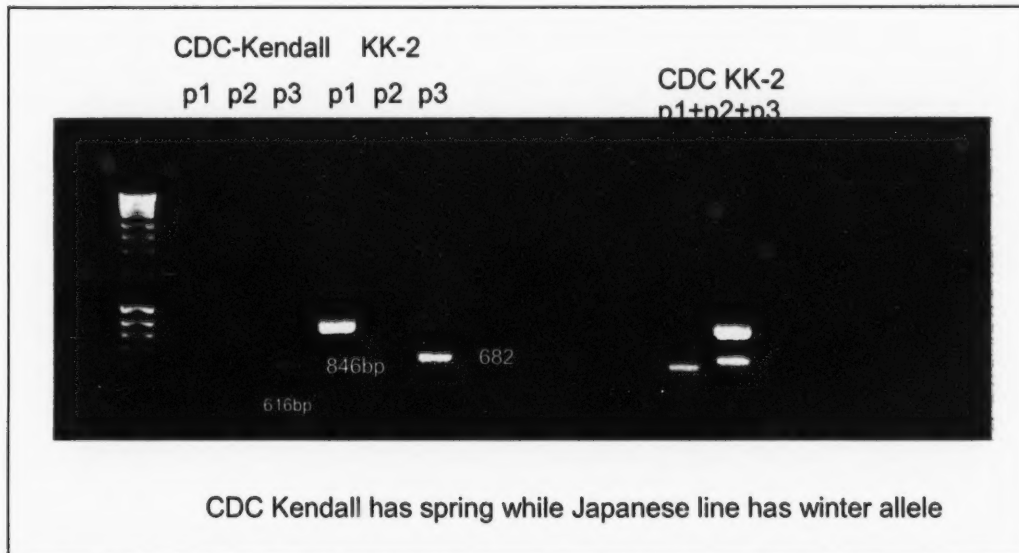


Fig. 6. Multiplex PCR-Based Markers Diagnostic for Spring and Winter growth habit in barley line CDC Kendall and Japanese line KK-2.

With primer 1 and 2 no product was obtained in case of CDC Kendall. PCR with primer 3 gave 616 bp product. In case of Japanese parent line primer 1 gave product of 846bp characteristic of winter growth habit and primer pair 3 gave 682bp product (Fig. 6).

Primer pair 1 and 3 were used for screening of breeding lines for summer/winter growth habit. Genomic DNA extracted from leaf tissue of similar age was used for this assay. Amplification of predicted amplicons of expected size from different breeding lines of this cross was achieved using an annealing temperature of 65°C, in the absence of GC-Rich buffer, with concentrations for each forward and reverse primer of 0.75 μ M (pair 1), 0.38 μ M (pair 2), and 1.0 μ M (pair 3).

In F-5 population of CDC x KK-2 out of eighty eight lines, 30 showed marker for spring habit and rest 58 are showed marker for winter habit. Forty lines out of sixty four single seed descent population showed spring and 24 showed winter marker. Granule size character was not found to be linked with either of the two as selected breeding lines showed ratio of 1:1 for spring and winter growth habit.

The experiment concluded no correlation between the spring or winter growth habits to granule size distribution trait.

Granule size distribution in Japanese line x CDC Clyde and Japanese line x CDC Copeland

The breeding lines obtained from this cross in 2009 field trial were analysed for granule size distribution with the aid of Malvern Master Sizer analyzer. Seeds for starch extraction were selected on random basis. Starch was extracted with same modified protocol as used in previous trials and 40 mg of starch was analysed on Master Sizer Analyser.

Breeding lines obtained from Japanese line x CDC Clyde (33) and from Japanese line x CDC Copeland showed clear segregation for granule size distribution. CDC Clyde had 12-14% B granule content while CDC Copeland showed 6-8% B- granule content (Fig. 7). The Japanese lines used in these two field trials showed 2-4% B-granule content. Atleast four lines in CDC Clyde x Japanese line and 8 in CDC Copeland x Japanese line showed B granule content either lower or similar to Japanese line (Fig. 7).

These 12 lines will be repeated in 2010 to confirm the results.

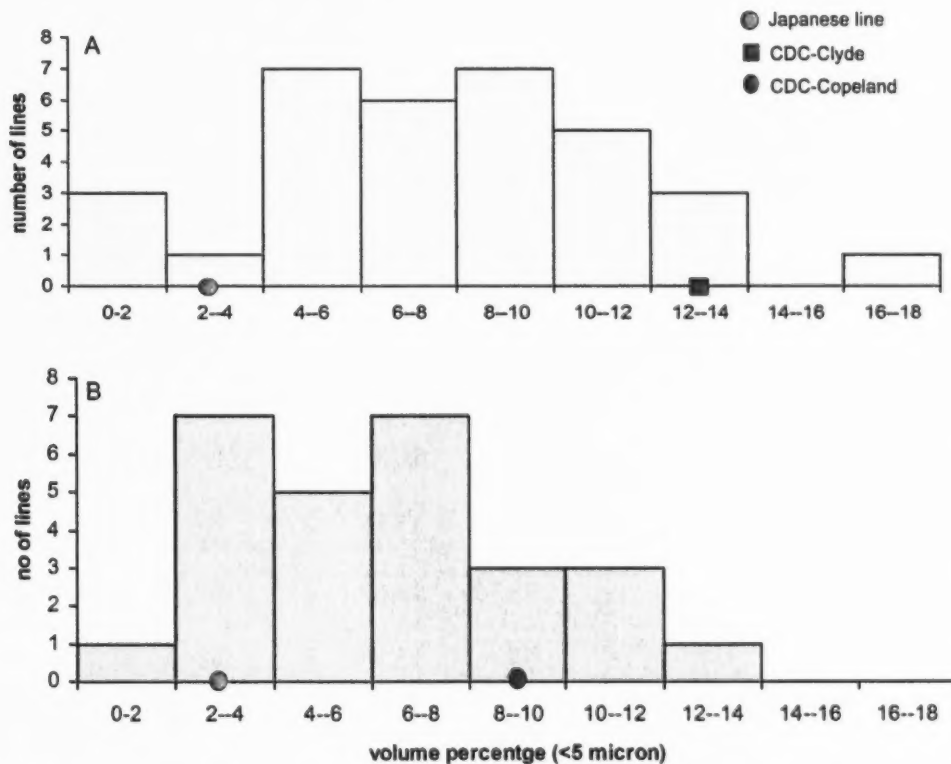


Fig. 7. Segregation of starch granule size <5 μ volume percentage in Japanese line x CDC-Clyde (A) and Japanese line x CDC Copeland population

Analysis of morphological and biochemical characters in breeding lines derived from CDC-Kendall x Japanese line to correlate with granule size trait:

From the total of eight lines selected from F-5 bulk population three were obtained from the 2009 field trial. These three lines were hulled, 70-76 cm in height, two row spike and awn length ranging 14-16 cm. Only one (line 25) out of selected eight lines showed morphological similarity with Japanese parent in terms of plant height, awn length and absence of hull.

These three (43, 47, 66) lines were used again for starch extraction and granule size distribution analysis (Table 3). The data obtained from this trial confirmed previous results.

In the case of 14 lines selected from F-5 single seed descent population, seven were obtained in the 2009 field trial and four of them still confirm the previous results (Table 3). Five lines out of fourteen were hullless, while rest were hulled.

Table 3. Morphological and biochemical features studied in selected F-5 bulk breeding lines of CDC-Kendall x Japanese line. Confirmation of results in 2009 field trial is indicated in the column labeled 'repeat'.

Breeding Line	B granule %	repeat	66 kD pro	Height (cm)	awn length (cm)	no of rows	h/hl	amylose
25	5.52	NA	-	44.4	8.16	2	hl	NA
26	5.71	NA	-	69.83	18.16	2	hl	NA
42	6.92	NA	p	76.6	14.33	2	h	25.13
43	4.26	y	-	77	14.1	2	h	26.8
46	6.54	NA	p	79.16	16.83	2	h	27.19
47	3.8	y	-	70.16	15.16	2	h	26.47
66	5.88	y	-	75.33	15.66	2	h	26.41
75	6.89	NA	-	67	15.16	2	hl	26

NA-Not available in 2009 field trial repeat

y-repeated and confirmed in 2009 trial

n-repeated but varied in 2009 trial

p-present

h-hulled

hl-hull less

Table 4. Morphological and biochemical features studied in selected F₅ Single head selected breeding lines of CDC-Kendall x Japanese line. Confirmation of results in 2009 field trial are indicated in the column labeled repeat

Breeding Line	B granule %	Field rep	66 kD pro	plant height	awn length	no of rows	h/hl	amylose
112	5.79	n	p	58.5	16.33	2	hl	22.12
116	4.81	n	p	37.83	5.2	2	hl	24.19
120	5.86	y	—	47	12.6	6	hl	NA
123	5.98	NA	—	70.16	13.83	6	h	23.53
126	5.85	NA	—	69.66	15	2	hl	25.52
135	7.41	n	—	66.66	20	2	h	NA
139	7.14	NA	—	66.6	20	2	h	25.74
147	6.18	NA	—	41.66	12	2	h	23.1
152	5.73	y	—	47.33	11.5	6	hl	24.37
161	4.21	y	p	44.5	7.4	6	h	23.02
168	3.27	NA	—	63	12.83	6	h	NA
170	4.24	NA	—	47.16	14.66	6	h	21.04
171	5.42	NA	p	73.5	17.66	2	h	24.9
173	3.25	y	—	40.83	9.96	6	h	25.24

NA-Not available in 2009 field trial repeat

y-repeated and confirmed in 2009 trial

n-repeated but varied in 2009 trial

p-present

h-hulled

hl-hull less

None of the features showed strong correlation with granule size distribution.

Objective 3

Develop targeted molecular markers for starch granule size distribution based on molecular analyses of natural variants and use a doubled haploid barley population (Steptoe x Morex or Harrington x TR 306) to identify novel QTL associated with starch granule development in barley.

As a first step to elucidate the molecular mechanism underlying starch granule development in barley, we conducted two experiments. In the first experiment we estimated the number of small B-type starch granules in the six barley genotypes described in Table 3. Barley grains were harvested at 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 30 and 40 days post-anthesis (dpa). Starch was isolated using the procedures described before (Demeke et al., 1997). The large (A-type) and small (B-type) starch granule number was determined using an image analysis system. All the starch granules at day 6 were less than 10 μm , thus classified as small starch granules. These starch granules grew larger and formed A-type granules ($= 10\mu\text{m}$), thus reducing the percent B-type granules to a low level around 10-16 dpa (Fig. 8). As expected, the number of small granules decreased to less than 20% at day 12 in all lines (Båga et al., 2000; Peng et al., 2000) except in Finnish 2 line, where it remained steady till day 14 and then decreased slightly to 70% on day 16. At day 14, the number of small starch granules started to increase in all lines to more than 80% at maturity. However, in the Japanese line, the B-type starch granule concentration reached only to 66% (Fig 8) thus confirming the results in Table 1.

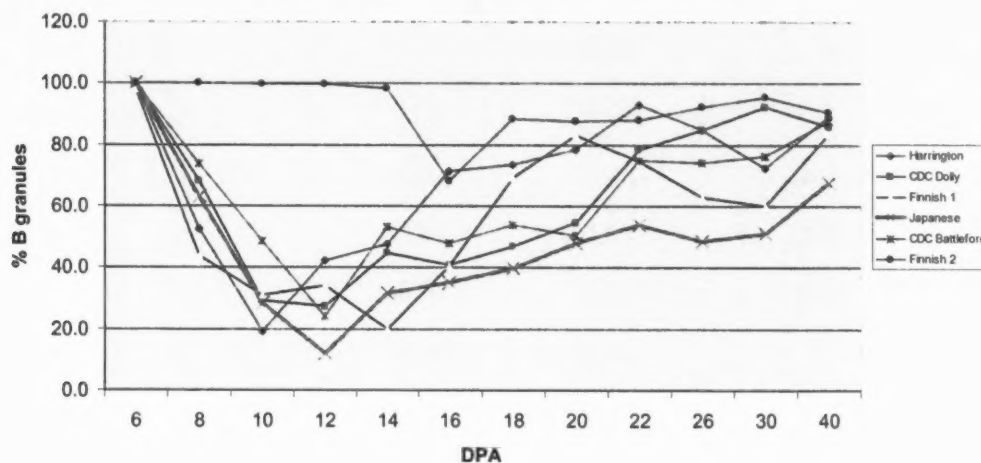


Fig. 8. Concentration of small starch granules ($= 10\mu\text{m}$) at different stages of grain development in selected barley varieties.

To study if there is a difference in starch structure between the two types of starch granules, we compared the total amylose concentration in the six starches selected from the Kernen field trial. Starch was isolated from mature seeds and amylose concentration was determined using a HPLC system as described before (Demeke et al., 1999). However, the amylose concentration in total starch did not show any significant difference between the lines tested (Table 3).

Table 3: Total amylose concentration in field-tested barley lines.

Location	Line	Total amylose
Kernen	CDC Battleford	26.2 %
Kernen	CDC Dolly	28.3 %
Kernen	Harrington	27.2 %
Kernen	Finnish line 1	27.9 %
Kernen	Finnish line 2	25.5 %
Kernen	Japanese line	26.6 %

The starch was further separated into large A-type and small B-type starch granules and amylose concentration was determined for each granule fraction. Results from control lines (CDC Battleford, CDC Dolly and Harrington) showed that A-type starch granules have 2-4% more amylose than B-type starch granules (Table 3), similar to finding in the literature (Ao and Jane, 2007). However, the Japanese line did not show any difference in amylose concentration between the small and large granules, in which ~26% amylose was produced (Table 4). Thus, the amylose/amylopectin ratio in small granules of the Japanese line differs from barley starches in general.

Table 4: Amylose concentration in the large and small starch granules in selected barley lines (n=number of samples analysed)

Genotypes	n	Percent amylose		
		A	B	A-B
CDC Battleford	6	26.0±1.1	22.8±1.7	3.2±1.4
CDC Dolly	3	26.6±1.0	22.7±2.8	3.9±3.7
Harrington	4	25.2±1.2	22.4±2.1	2.8±1.5
Finnish 1	4	24.6±1.0	21.5±1.7	3.1±2.2
Finnish 2	4	25.2±1.0	22.7±0.6	2.5±1.2
Japanese line	6	26.2±1.1	26.1±0.9	0.1±0.7

These results suggest that the small B-type starch granules in the Japanese line could be A-type granules that did not develop into large granules and no true B-type starch granules are formed in the Japanese line. Alternatively, synthesis of amylose and amylopectin in the B-type granules of the Japanese line may have been affected by a mutation, e.g. a starch biosynthetic enzyme, causing altered amylose /amylopectin ratios and packaging of glucan polymers affecting the

granule size. Further experiments will be conducted in this year to confirm these results and determine the molecular mechanism for this interesting observation.

These results from the first year of study and those reported in milestone 2 concur that the small B-type starch granules in the Japanese line could be A-type granules that did not develop into large granules and no true B-type starch granules are formed in the Japanese line. Alternatively, synthesis of amylose and amylopectin in the B-type granules of the Japanese line may have been affected by a mutation, e.g. a starch biosynthetic enzyme, causing altered amylose /amylopectin ratios and packaging of glucan polymers affecting the granule size. In the year under review, we conducted experiments to show the association of amylose concentration with starch granule size. We made use of some of the barley lines with changed carbohydrate profiles to conduct this study. Ten two -row spring barley lines were analyzed for carbohydrate profiles. The genotypes included in this study were CDC McGwire, CDC Rattan, CDC Fibar, CDC Candle and CDC Alamo. The rest were SH99250, SH99073, SB94893, SB94912 and Waxy Betzes. CDC McGwire has a normal starch while CDC Rattan, CDC Fibar, CDC Candle, CDC Alamo, SB94912 and Waxy Betzes are waxy (0-<5% starch amylose). SH99250, SH99073 and SB94893 have been reported to have elevated grain starch amylose (=35%).

Standard protocols were used to analyze total starch, amylose concentration, starch granule size, beta-glucan, dietary fiber, protein and fat concentrations. However, for this report we will present the results related to total starch, amylose and starch granule size distribution.

Total Starch concentration:

Total starch content varied among different barley lines and ranged from 58.1% to 72.2% (Table 2). CDC McGwire had the highest percent total starch concentration 72.2%, while CDC Fibar recorded the lowest, 58.1%. SH 99073, SB 94912, SH 99250 and SB99893 had total starch content of 59.7%, 61%, 61.1% and 61.9% respectively. Percent starch content in the other barley lines were CDC Alamo 64.2%, CDC Rattan 65.6%, Waxy Betzes 66.5% and CDC Candle 68.5% (Table 5).

Table 5. Barley grain composition and starch granule sizes

Barley Lines	Carbohydrates				Non- starch Carbohydrates		
	% Starch concentration	Amylose concentration by HPLC	Beta glucn by Calcofluor	Beta glucan by Megazyme	Total Dietary Fiber	Protein	Fat
CDC McGwire	72.2 ± 0.6	25.8 ± 0.7	5.8 ± 0.4	5.1 ± 0.4	11.8 ± 1.1	13.5 ± 0	3.04 ± 0.4
CDC Rattan	65.6 ± 1.1	0 ± 0	8.6 ± 0.1	7.4 ± 0.1	14.2 ± 0.8	15 ± 0.2	4.23 ± 0.6
CDC Fibar	58.1 ± 1.8	0 ± 0	13.3 ± 0.1	9.7 ± 0.4	17.5 ± 0.6	16.6 ± 0.2	3.6 ± 0.1
SH 99250	61.1 ± 2.2	38.5 ± 0.3	11.3 ± 1.7	7.9 ± 0.6	17.4 ± 0.7	14.5 ± 0.1	3.4 ± 0.2
SH 99073	59.7 ± 0.6	40.8 ± 1.1	9.7 ± 0.5	8.5 ± 0.3	18.2 ± 0.6	14.8 ± 0	3.3 ± 0.2
SB 94893	61.9 ± 0.2	38 ± 0.1	8.9 ± 0.3	7.9 ± 0.1	16.0 ± 0.7	15 ± 0	3.2 ± 0.1
CDC Candle	68.5 ± 0.9	4.5 ± 0.4	6.8 ± 0.2	6.9 ± 0.1	13.6 ± 0.5	13.6 ± 0	3.4 ± 0.1
Waxy Betzes	66.5 ± 0.7	3.9 ± 0.1	8.7 ± 0.4	7.1 ± 0.3	15.1 ± 0.9	14.2 ± 0.1	3.4 ± 0.5
SB 94912	61.0 ± 0.7	1.6 ± 0.5	9.8 ± 0.2	8.2 ± 0.2	15.0 ± 0.2	15.2 ± 0.1	3.8 ± 0.2
CDC Alamo	64.2 ± 0.2	0 ± 0	8.7 ± 0.4	7.3 ± 0.1	13.7 ± 0.4	15 ± 0	4.4 ± 0.3

Percent amylose concentration:

Percent amylose content in barley lines as determined by the size exclusion-HPLC (SE-HPLC) in 99% DMSO was from a low of 0% to a high of 40.8% (Table 5). Six barley lines CDC Rattan, CDC Fibar, CDC Candle Waxy Betzes, SB 94912 and CDC Alamo had percent amylose content less than 5% (waxy lines).

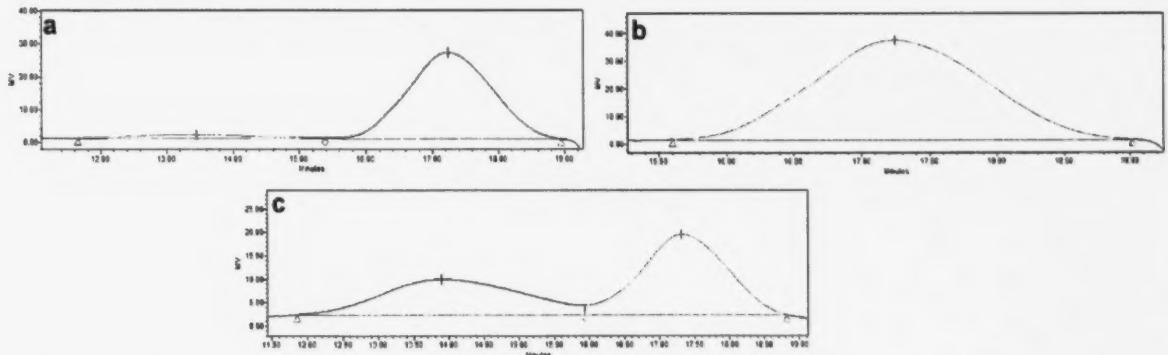


Fig. 9. Amylose concentration determined by HPSEC method in barley lines differing in waxy locus. a represent partial waxy, b waxy and c, control barley line

There was no amylose detected by the HPLC method (0%) in CDC Rattan, CDC Fibar and CDC Alamo (Figure 2). However percent amylose values for CDC Candle, Waxy Betzes, and SB 94912 were greater than 0% but less than 5%. CDC Candle had 4.5% while Waxy Betzes, and SB 94912 recorded 3.9% and 1.6% respectively. CDC McGwire had 25.8% amylose (normal). Three barley lines were classified as high amylose lines because their percent amylose content was more than 35%. These amylose contents were higher than the normal (CDC McGwire). The high amylose values were from 38% to 40.8%. SH99073 had the highest starch amylose (40.8%) followed by SH99250, 38.5% and SB 94893, 38%. All the high amylose lines also had lower starch content compared to the normal or waxy lines with CDC Fibar (waxy) being the exception. CDC Fibar was waxy jet recorded the lowest starch content.

Percent starch granule sizes < 10µm:

All genotypes showed a typical bimodal particle size distribution. Percent granule sizes < 10µm determined were high for CDC McGwire and all the waxy genotypes. Starch granules < 10µm in CDC McGwire (normal) was 89% while among the waxy genotypes CDC Rattan had the highest smaller granules, 97.5%. The high amylose genotypes were low in small starch granules with values ranging from 40.8% to 55.8% for SH99250 SH99073 and SB94893 respectively (Fig. 10 A-J)

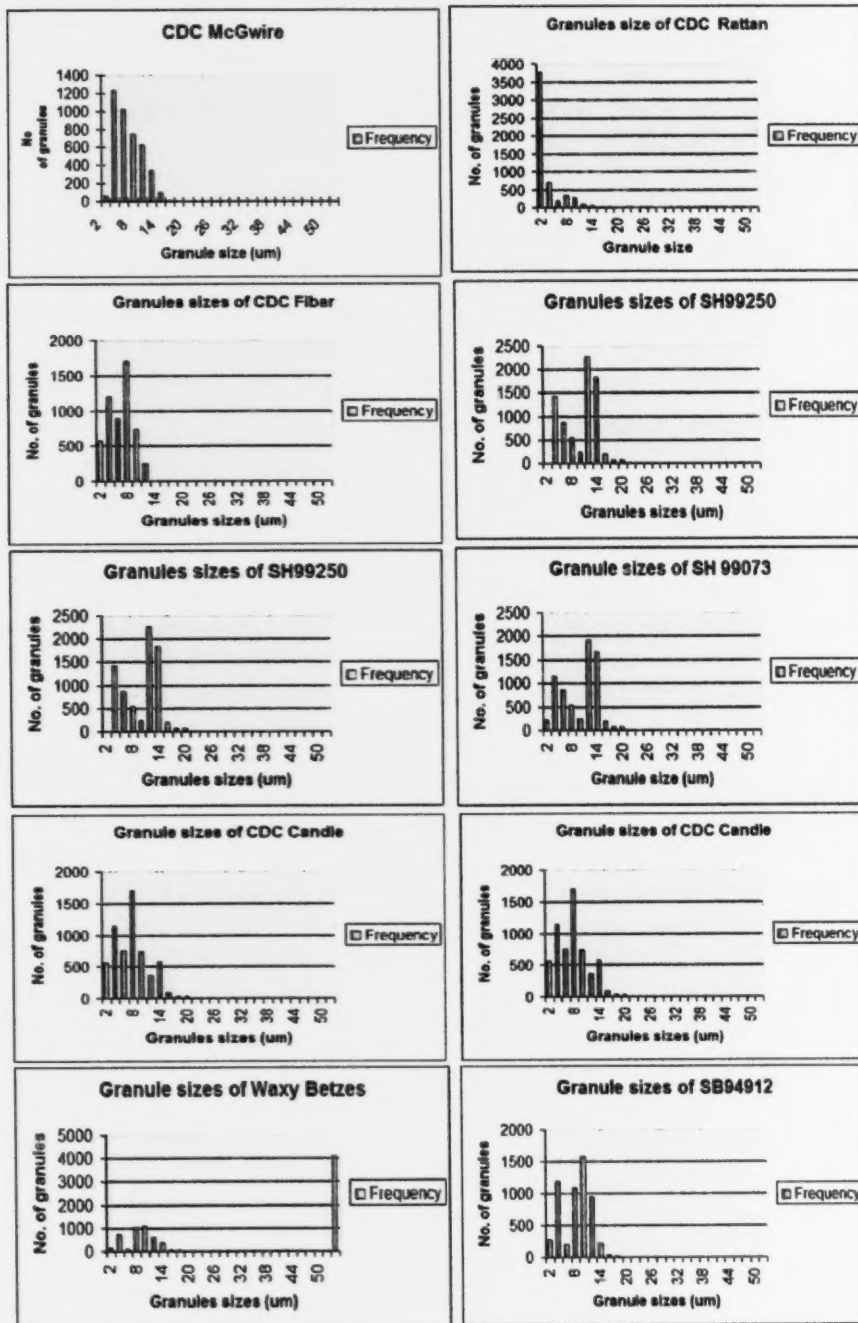


Fig 10. Granule sizes distribution for barley genotypes. A, CDC McGwire B, CDC Rattan; C, CDC Fibar; D, SH99250; E, SH99073; F, SB94893; G, CDC Candle; H, Waxy Betzes; I, SB94912 and J, CDC Alamo.

The main conclusion from this part of work was that increased amylose concentration in barley genotypes was associated with decrease in the small starch granules (B-type) in barley. This confirmed the results reported in the May 2006-June 2007 report, that the Japanese line which had increased A-type granules there was no significant difference in amylose concentration between the A- and B-type starch granules. This also concurs with the hypothesis put forth by Ao and Jane (2007).

Variation in starch granule bound starch biosynthetic enzymes

The main objective of this work is to study the variation in starch biosynthetic enzymes between the barley genotypes that differ in amylose concentration and starch granule size distribution.

SDS-PAGE gels for starch granule proteins:

SDS-PAGE gel electrophoresis was used to investigate the composition of starch biosynthetic enzymes in the barley genotypes. Gel consisted of 10% and 20% resolving for SSII and GBSS1 polypeptides respectively. The 60kDa protein was completely absent or weak in lanes 2, 4, 5 and 10 which are all waxy barley genotypes (Fig 11). However lane 6 (CDC Alamo) though waxy shows a 60kDa protein as previously reported (Patron et al., 2002) while in lane 3 (CDC Fibar) a waxy genotype, there is currently no report on the presence of the 60kDa protein. Lanes 7, 8 and 9 are the high amylose lines, SH99250, SH99073 and SB94893 respectively.

The main conclusion from this experiment is that in all the three barley lines, with increased amylose concentration and reduced B-type starch granules, both the starch synthase II and starch branching enzyme II (SBE II) show increased amounts as compared to other barley lines with normal amylose and starch granule size distribution. This does not agree with the current literature where high amylose concentration is either correlated with reduced SSII in barley sex 6 mutants (Morell et al, 2003) or in transgenic wheat with reduced SBEII as a result of RNAi inhibition (Regina et al., 2006). This needs further investigation. In the future work plan we will DNA sequence the SSII and III and SBE II and I alleles and study their expression from all the barley lines during grain development to understand the role of these genes in starch granule size distribution.

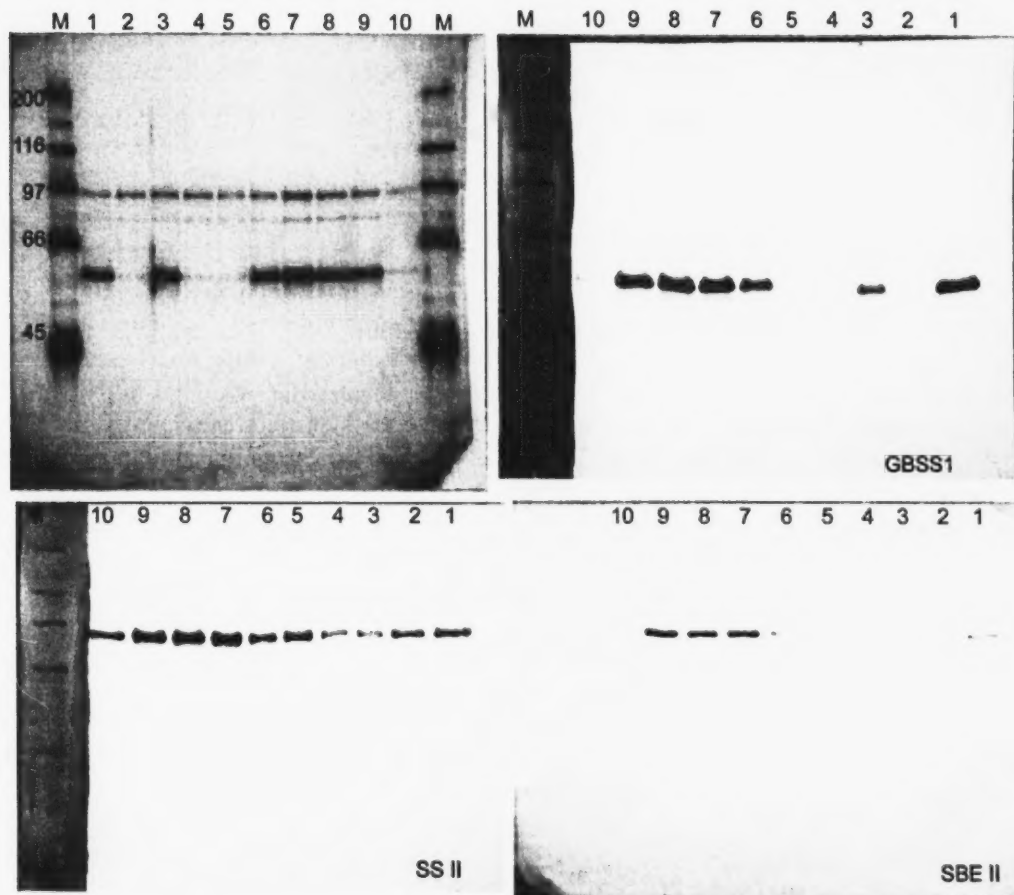


Fig. 11. Electrophoretic analysis/ silver stain detection and immuno-reaction of endosperm starch granule proteins from barley genotypes. (a) SDS-PAGE gel showing GBSS I polypeptides. M is marker, lane 1 CDC McGwire; 2. CDC Rattan; 3, CDC Fibar; 4, CDC Candle; 5, Waxy Betzes; 6 CDC Alamo; 7 SH99250; 8, SH99073; 9 SB94893 and 10 CDC Alamo. (b) The immuno-reaction of barley starch granule proteins (GBSS1) with polyclonal antibodies raised against wheat GBSS1. c). SDS-PAGE gel showing SS11 polypeptides. M is marker, lane 1 CDC McGwire; 2. CDC Rattan; 3, CDC Fibar; 4, CDC Candle; 5, Waxy Betzes; 6 CDC Alamo; 7 SH99250; 8, SH99073; 9 SB94893 and 10 CDC Alamo. (d) The immuno-reaction of barley starch granule proteins (SS II) with polyclonal antibodies raised against wheat SSII. (e) The immuno-reaction of barley starch granule proteins (SBE II) with polyclonal antibodies raised against wheat SBE II.

The main conclusion from this experiment is that in all the three barley lines, with increased amylose concentration and reduced B-type starch granules, both the starch synthase II and starch branching enzyme II (SBE II) show increased amounts as compared to other barley lines with

normal amylose and starch granule size distribution. This does not agree with the current literature where high amylose concentration is either correlated with reduced SS II in barley sex 6 mutants (Morell et al, 2003) or in transgenic wheat with reduced SBE II as a result of RNAi inhibition (Regina et al., 2006). This needs further investigation.

Biochemical analysis of polypeptides associated with starch granules of CDC Kendall x Japanese line KK-2

Protein extraction and analysis was carried out for starch associated proteins using 20% and 10% SDS-PAGE. As the Japanese parent used in this study has a mutation which may have its effects on starch biosynthesis. PAGE profile of CDC-Kendall and the Japanese parent was compared and two polypeptides were found to be unique for the Japanese parent (66.6 and 53.2 kD) (Fig. 12). Immunoblot analysis by using antibodies of known starch biosynthesis enzymes (GBSS, SSI, SSII, SBE I and SBE II) was carried out for identification of these unique polypeptides present in Japanese parent. None of these antibodies reacted with the two polypeptides identified in KK2 starch granule proteins.

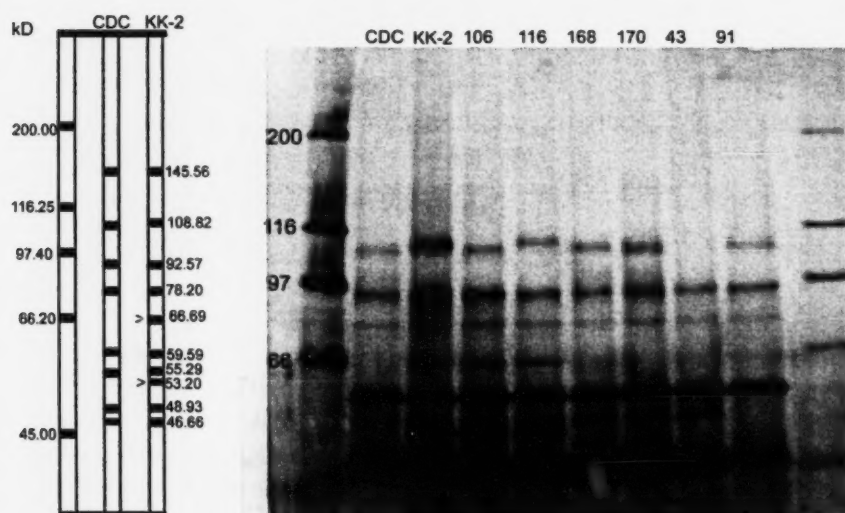


Fig. 12. Electrophoretic analysis/ silver stain detection of endosperm starch granule proteins CDC Kendall, Japanese line and some of the selected breeding lines with reduced B granule content.

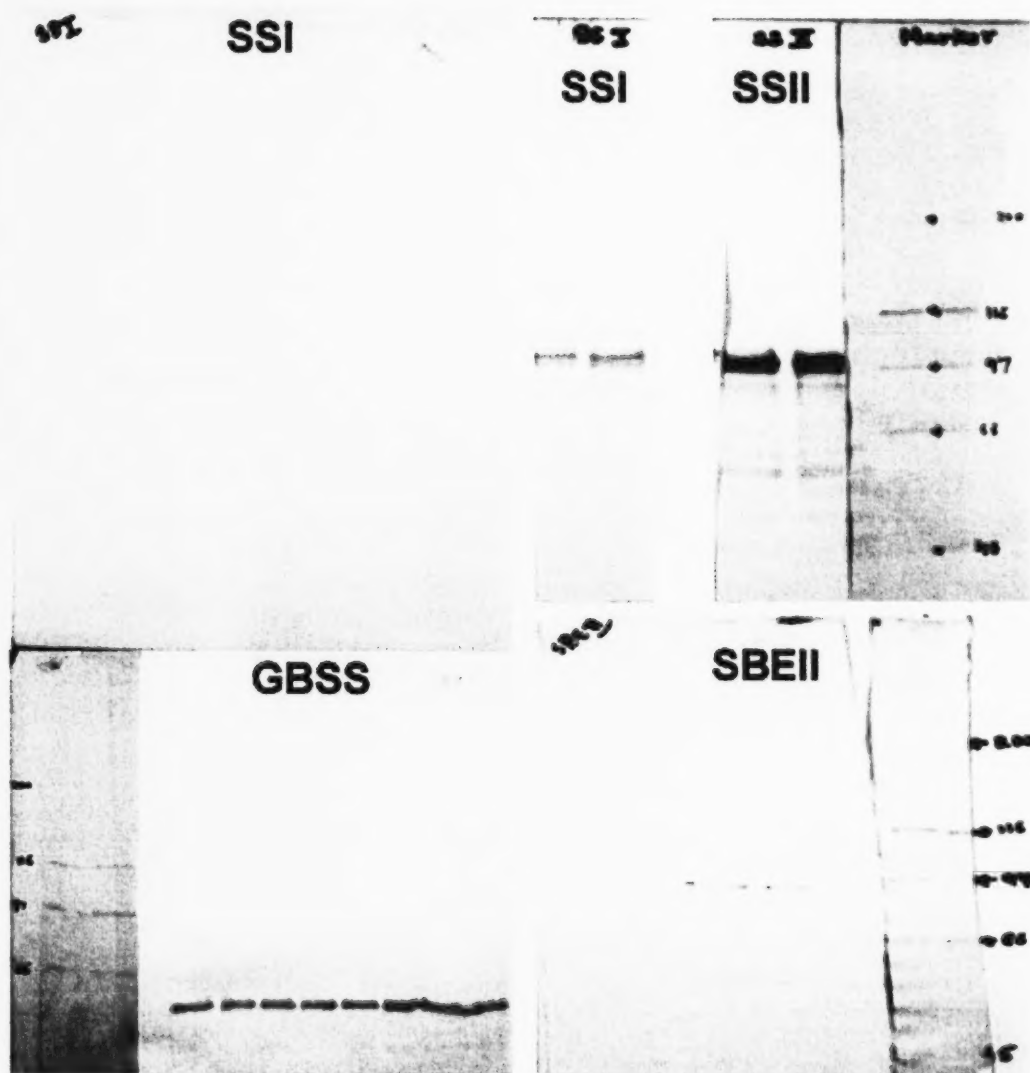


Fig. 13. Immunochemical detection of SSI, SSII, GBSS and SBE II in starch granule bound proteins in CDC Kendall and Japanese line.

Starch biosynthesis related proteins in soluble and granule bound portion in developing endosperm of CDC-Kendall and Japanese line

SDS-PAGE profile of CDC-Kendall and Japanese line soluble fraction of developing endosperm (20dpa) showed two major differences at approximately 156 and 87 kD (Fig. 14). The polypeptides corresponding to these molecular weight appeared comparatively more intense in

case of CDC- Kendall. In the granule bound fraction no major difference other than increase of 2kD (108kD) in Japanese line in comparison to CDC-Kendall (106kD) was observed.

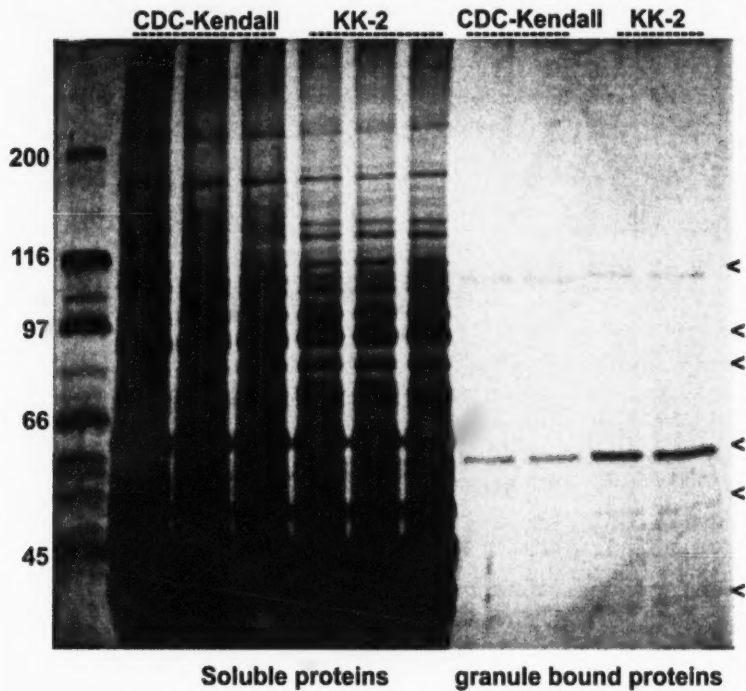


Fig. 14. Soluble and granule bound polypeptides in the developing endosperm (late milky stage) of CDC-Kendall and Japanese line KK-2.

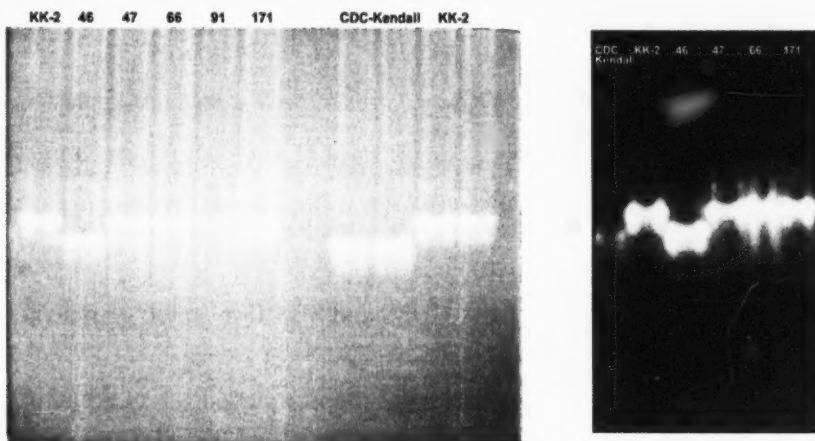


Fig. 15. Phosphorylase and amylolytic activity in Soluble fraction of developing endosperm of CDC Kendall, Japanese line (KK-2) and selected breeding lines.

Phosphorylase activity and amylolytic enzyme activity Native PAGE analysis (Fig. 15) showed major changes between parental lines and strong correlation between the profile of Japanese and selected breeding lines. Whether the changed phosphorylase and amylolytic isozymes pattern contribute to altered granule size phenotype observed in this study needs further clarification and proof.

Objective 4

Use a doubled haploid barley population (Steptoe x Morex or Harrington x TR 306) to identify novel QTL associated with starch granule development in barley

The main objective of this part is to identify the barley chromosomal regions associated with starch granule size distribution. Preliminary results with phenotyping the parents of these crosses for starch granule size showed that no significant variation in the trait could be observed. However, the results based on the experiments described above clearly showed that amylose concentration and starch granule size are related. The results with the identified Japanese lines, as well as three barley breeding lines showed that increased amylose concentration resulted in decreased numbers of small B-type starch granules. In another project, in a wheat mapping population, we had identified a novel quantitative trait locus (QTL) on chromosome 2 which is associated with increased amylose concentration and starch swelling power, two traits closely related to starch granule size. Therefore, to expedite the project objectives, we chose to concentrate on this QTL in wheat genome to determine DNA-based markers for starch granule size distribution. The results obtained to map the genomic regions associated with amylose concentration/starch granule size are summarized below.

A wheat DH population segregating for amylose concentration was phenotyped for amylose concentration by HPLC method (Demeke et al., 1999) and starch swelling power, a flour functional trait related to starch properties (Ansari et al. 2010). Ninety two lines were genotyped using 2500 Diversity Array (DArT) polymorphic markers to develop a genetic map. Bioinformatic analyses was carried out to identify the expressed sequenced tags (EST) mapping to the QTL region. An EST corresponding to starch synthase 3 was mapped to the chromosome 2 associated with high amylose concentration. Subsequently using the DNA sequence of starch synthase III, oligonucleotide primers were designed and the same starch synthase 3 was mapped to chromosome 2 using a DH population Norstarx winter Manitou and Norstar X Cappelle Desprez (Båga et al 2007) and a comparative genetic map was developed (Fig. 16).

The location of a starch synthase III on chromosome 2 has not been reported before. Therefore, the results suggest a novel form of SS III related to amylose concentration discovered in this project. We used a wheat BAC library to isolate complete SS III genomic clones from the wheat A (2AL) and B (2BL) genomes. The BAC clones have been DNA sequenced.

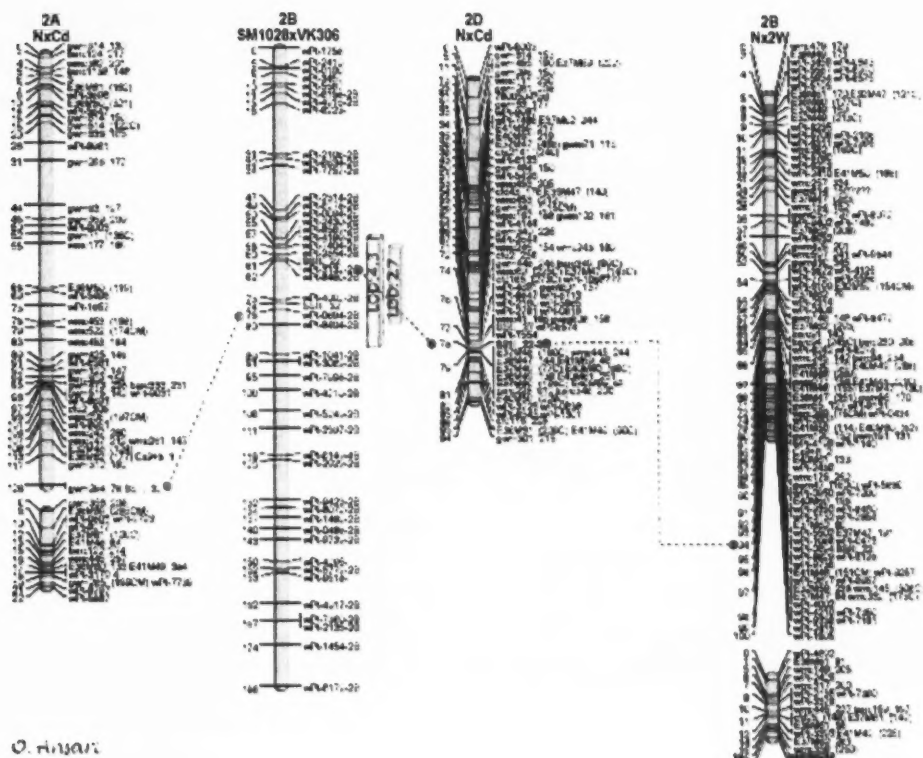


Fig. 16. Location of SSIII alleles on different genomes in different populations. Bars are approximate location of QTL on OA24 (SM1028 $\times$ VK306) population. N $\times$ 2W: Norstar $\times$ winter Manitou and N $\times$ Cd: Norstar $\times$ Capel-Deprez.

There is considerable synteny between the cereal genomes and we are confident that the markers developed using this approach will be valid for wheat, barley and the other two cereals, rye and triticale that show bimodal starch granule size distribution.

CONCLUSIONS AND RECOMMENDATION

Starch granule size, which forms an important trait in determining barley end use, is affected by many internal and external factors during starch biosynthesis. In the population derived from CDC-Kendall and Japanese clear segregation of the trait was observed. KK-2 and some breeding lines show volume percentage of different size granules skewed more towards medium size granules. We speculate that these medium size starch granules are large A-type starch granules which failed to grow to the actual size.

The study focused on barley lines differing in waxy locus indicated that high amylose concentration is related to decrease in B-type granules or increase in A-type starch granule. This is associated with increased accumulation of starch synthase II and starch branching enzyme II polypeptides in starch granules. Two new starch granule polypeptides which may be associated with altered granule size distribution present only in the Japanese line KK-2 have been identified.

We have taken a different approach to identify QTL for increased amylose concentration that is associated with decreased B-type starch granules or increased A-type starch granules. A novel starch synthase III allele has been shown to be associated with the identified QTL. The DNA sequencing of the gene has been completed. Data is being analyzed for developing DNA markers for use in marker assisted selection.

Major objective of this project to develop barley lines with reduced B-type starch granules has been achieved. The identified lines need to be optimized for growing in Saskatchewan conditions. This project has also resulted in several interesting observations in starch biosynthesis. The association of increased amylose concentration with reduced B-type starch granules needs to be studied further. Identification of two new starch granules polypeptides associated with granule size distribution need to be characterized for their use as molecular markers for starch granule size distribution. The identification of a new starch synthase III (SSIII) is a very interesting finding. The new SSIII when fully characterized will have far reaching implications to alter starch granule size and amylose concentration in wheat and barley.

ACKNOWLEDGEMENTS

Financial assistance for the project from Saskatchewan Ministry of Agriculture, Agriculture Development Fund, Canada Research Chairs, Canada Foundation for Innovation and NSERC is gratefully acknowledged. Help by Shelley Duncan and Doug Voth in growing F2, F5 lines is highly appreciated. AAFC-Saskatchewan Research Centre is gratefully acknowledged for use of their Malvern-Mastersizer.

REFERENCES

- Ao, Z. and Jane, J.-L. (2007) Characterization and modelling of the A- and B-granule starches of wheat, triticale and barley. *Carbohydrate Polymers* 67:46-55.
- Båga, M., Chodaparambil, S. V., Limin, A., Pecar M., Fowler D.B. and Chibbar R.N., (2007). Identification of Quantitative Trait Loci and Associated Candidate Genes for Low-Temperature Tolerance in Cold-Hardy Winter Wheat. *Functional & Integrative Genomics* 7:53-68.
- Båga, M., Nair, R.B., Repellin, A., Scoles GJ, and Chibbar R.N. (2000) Isolation of a cDNA encoding a granule-bound 152-kilodalton starch-branching enzyme in wheat. *Plant Physiol* 124: 253-263
- Borem, A., Mather, D.E., Rasmusson, D.C., Fulcher, R.G., and Hayes, P.M. (1999) Mapping quantitative trait loci for starch granule traits in barley. *J Cereal Sci* 29: 153-160.
- Cockram, J., Chiapparino, E., Taylor, S.A., Stamati, K., Donini, P., Laurie, D.A., and O'Sullivan, D.M. (2007a). Haplotype analysis of vernalization loci in European barley germplasm reveals novel VRN-H1 alleles and a predominant winter VRN-H1/VRN-H2 multi-locus haplotype. *Theor. Appl. Genet.* 115:993-1001.
- Cockram, J., Mackay, I.J., and O'Sullivan, D.M. (2007b). The role of double-stranded break repair in the creation of phenotypic diversity at cereal VRN1 loci. *Genetics* 177:1-5.
- Cockram, J., Norris, C., and O'Sullivan, D.M. (2009) PCR-based markers diagnostic for spring and winter seasonal growth habit in barley *Crop Sci.* 49: 403-410.
- Cockram, J., White, J., Leigh, F.J., Lea, V., Mackay, I.J., Laurie, D.A., Powell, W., and O'Sullivan, D.M. (2008). Association mapping of partitioning loci in barley. *BMC Genet.* 9:16.
- Davis, J. (1964). Disc electrophoresis. 11. Method and application to human serum proteins. *Annals of the New York Academy of Science* 121: 404-427.
- Demeke, T., Hucl P., Båga M., Caswell K., Leung N. and Chibbar R.N. (1999). Transgene inheritance and silencing in hexaploid wheat. *Theoretical and Applied Genetics* 99: 947-953.
- Demeke, T., Hucl P., Nair R.B., Nakamura T. and Chibbar R.N. (1997) Evaluation of Canadian and other wheats for waxy proteins. *Cereal Chem.* 74: 442-444.
- Demeke, T., P. Hucl, M. Båga, K. Caswell, N. Leung and R.N. Chibbar (1999). Transgene inheritance and silencing in hexaploid wheat. *Theoretical and Applied Genetics* 99(6):947-953.
- Fishman, M.L., Rodriguez, L. and Chau H.K. (1996). Molar masses and sizes of starches by high-performance size exclusion chromatography with on-line multiangle laser light scattering detection. *J. Agric. Food Chem.* 44: 3182-3188.
- Fu, D., P. Szűcs, Yan, L., Helguera, M., Skinner, J.S., von Zitzewitz, J., Hayes, P.M., and Dubcovsky, J. (2005). Large deletions within the first intron in VRN-1 are associated with spring growth habit in barley and wheat. *Mol. Genet. Genomics* 273:54-65.
- Gao, M. and Chibbar, R.N. (2000) Isolation, characterization, and expression analysis of starch synthase IIa cDNA from wheat (*Triticum aestivum* L.) *Genome* 43:768-775.
- Hizukuri, S., Takeda Y. and Yasuda M. (1981). Multi-branched nature of amylose and the action of debranching enzymes. *Carbohydr. Res.* 94: 205-213.
- Jane, J. and Chen, J.F. (1992) Effect of amylose molecular size and amylopectin branch chain

- length on paste properties of starch. *Cereal Chem.* 69: 60-65.
- Jane, J. and Shen, J.J. (1993) Internal structure of the potato starch granule revealed by chemical gelatinization. *Carbohydr. Res.* 247: 279-290.
- MacGregor, A.W. and Balance, D.L. (1980). Hydrolysis of large and small starch granules from normal and waxy barley cultivars by alfa-amylases from barley malt. *Cereal Chem.* 57: 397-402.
- McLeary, B.V., Solah, V. and Gibson, T.S. (1994) Quantitative measurement of total starch in cereal flours and products. *J Cereal Sci.* 20:51-58.
- Meredith P. (1981) Large and small starch granules in wheat - are they really different? *Starch* 33: 40-44.
- Morell, M.K., Kosar-Hashemi, B., Cmiel, M., Samuel, M.S., Chandler, P., Rahman, S., Buleon, A., Batey, I.L., Li, H. (2003). Barley *sex6* mutants lack starch synthase IIa activity and contain a starch with novel properties *Plant J.* 34: 173- 186
- Morrison, W.R. (1995) Starch lipids and how they relate to starch granule structure and functionality. *Cereal Foods World* 40: 437-446.
- Nishi, A., Nakamura, Y., Tanaka, N., and Hikaru, S. (2001). Biochemical and genetic analysis of amylose extender mutation in rice endosperm. *Plant Physiol.* 127: 459-472.
- Ansari, O., Baga, M., Chibbar, R. N., Sultana N, Howes, N. K. (2010) Analysis of starch swelling power in Australian breeding lines of hexaploid wheat (*Triticum aestivum* L.) *Field Crops Research* 115: 171-178.
- Pan, D.D. and Jane, J. (2000) Internal structure of normal maize starch granules revealed by chemical surface gelatinization. *Biomacromolecules.* 1:126-132.
- Peng, M., Gao, M., Baga, M., Hucl, P., and Chibbar R.N. (2000) Starch-branching enzymes preferentially associated with A-type starch granules in wheat endosperm. *Plant Physiol* 124:265-272.
- Pierce, J.S. (1991) "European Brewery Convention - Advances in Malting Barley", Barley and Malting Committee of the European Brewery Convention.
- Regina, A., Bird, A., Topping, D., Bowden, S., Freeman, J., Barsby, T., Kosar-Hashemi, B., Li, Z., Rahman, S., and Morell. M. (2006). High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. *Proc. Natl. Acad. Sci. U. S. A* 103:3546-3551.
- Szucs, P., Skinner, J.S., Karsai, I., Cuesta-Marcos, A., Haggard, K.G., Corey, A.E., Chen, T.H.H., and Hayes, P.M. (2007). Validation of the VRN-H2/VRN-H1 epistatic model in barley reveals that intron length variation in VRN-H1 may account for a continuum of vernalization sensitivity. *Mol. Genet. Genomics* 277:249-261.
- Takeda Y, Shirasaka K. and Hizukuri S. (1984). Examination of the purity and structure of amylose by gel-permeation chromatography. *Carbohydr. Res.* 132: 83-92.
- Takeda, Y. and Hizukuri, S. (1987). Structure of branched molecules of amyloses of various origins, and molar fractions of branched and unbranched molecules. *Carbohydr. Res.*, 165: 139-145.
- von Zitzewitz, J., Szucs P., Dubcovsky J., Yan L., Francia E., Pecchioni, N., Casas, A., Chen, T.H.H., Hayes, P.M., and Skinner J. (2005). Molecular and structural characterization of barley vernalization genes. *Plant Mol. Biol.* 59:449-467.
- Yoo, S. and Jane, J. (2002). Molecular weights and gyration radii of amylopectins determined by high-performance size-exclusion chromatography equipped with multi-angle laser light scattering and refractive index detection. *Carbohydr. Polym.* 49: 307-314.
- You, S. G. and Izydorczyk, M.S. (2002). Molecular characteristics of barley starches with

variable amylose content. Carbohydrate Polymers. 49: 33-42.

Zhao, X.C., Sharp, P.J. (1996). An improved 1-D SDS-PAGE method for the identification of three bread wheat 'waxy' proteins. Journal of Cereal Science 23:191-193

Zobel, H.F. (1988). Molecules to granules: A comprehensive starch review. Starch/Stärke. 44: 44-50.

Other – (e.g. extension meetings; papers produced; conference presentations made; personnel involved; equipment bought; photos; project materials developed; expense statement)

Papers produced and conference presentations made:

Journal Publications:

Maley, J, Asare, EK, Båga, M, Rossnagel, BG, Chibbar, RN and Sammynaiken R. (2010) Imaging starch biopolymers using atomic force microscopy. *Starch/Staerke* (submitted).

Conference Proceedings (full)

Ganeshan, S, Båga, M, Jaiswal, S, Ng, SH, Chibbar, R, Hucl, P, Rossnagel, BG, and Chibbar, RN. (2010) Genetic modification of cereal grain carbohydrates for food, feed and improved human health. In: *Quality Science for the Country – Proceedings 59th Australian Cereal Chemistry Conference*, Waga Waga, New South Wales, Australia, September 27-30, 2009.

Chibbar, RN, Båga, M, Ganeshan, S, Hucl, P, Li, J, Singh, R, Asare, EK, Verma, B, and Rossnagel, BG. (2008) Genomic strategies to modify grain carbohydrates to diversify utilization of cereal grains. In: *Sustainable Agriculture for Food, Bioenergy and Livelihood Security*, (eds. R.K. Behl, D.P. Singh, V.S. Tomar, M.S. Bhale, D. Khare, and S.D. Upadhyaya) Agrobios (international), Jodhpur, India, pp. 9-20.

Invited Conference Presentations:

Chibbar, RN. (2009) Genetic modification of cereal grain carbohydrates for food, feed and improved human health. *59th Australian Cereal Chemistry Conference – Quality Science for the Country*, Charles Stuart University, Waga Waga, NSW, Australia, September 27-30, 2009 (keynote lecture).

Båga, M, Rossnagel, BG, and Chibbar, RN. (2006) Molecular characterization of the genetic basis for starch granule size distribution in barley. *American Society of Brewing Chemists Annual Meeting*, La Quinta Resort and Club, La Quinta, California, USA, June 17-21, 2006.

Poster Presentations - Conferences

Jaiswal, S, Båga, M, Rossnagel, BG, and Chibbar, RN. (2009) Characterization of barley lines with altered starch granule size distribution. *Wheat Science Dynamics: Challenges and Opportunities, International Wheat Quality Conference – IV*, Saskatoon, SK, Canada, June 2-6, 2009, pp. 82.

Asare, EK, Båga, M, Ganeshan, S, Rossnagel, BG, and Chibbar, RN. (2009) Effect of changes in starch amylose concentration on other grain storage components in some barley (*Hordeum vulgare*) lines. *Wheat Science Dynamics: Challenges and Opportunities, International Wheat Quality Conference – IV*, Saskatoon, SK, Canada, June 2-6, 2009, pp. 83.

Asare, EK, Rossnagel, BG, and Chibbar, RN. (2009) Grain complex carbohydrates and starch granule composition influence *in vitro* enzymatic hydrolysis of grain starch. *Wheat*

Science Dynamics: Challenges and Opportunities, International Wheat Quality Conference – IV, Saskatoon, SK, Canada, June 2-6, 2009, pp. 116.

Ansari, O, Båga, M, and Chibbar, RN. (2009) Identification and characterization of a new starch synthase gene (*TASSIIIB*) isoform expressed in wheat (*Triticum aestivum* L.) endosperm. The 16th Annual Life and Health Sciences Research Conference, University of Saskatchewan, Saskatoon, SK, Canada, March 13, 2009, pp. 63. **(Winner second place award in the Plant Sciences section)**

Asare, E, Båga, M, Ganeshan, S, Rossnagel, BG, and Chibbar, RN. (2008) Characterization of barley (*Hordeum vulgare* L.) with altered carbohydrate composition. The 16th Annual Life and Health Sciences Research Conference, University of Saskatchewan, Saskatoon, SK, Canada, March 13, 2009, pp. 64.

Jaiswal, S, Ahuja, G, Båga, M, and Chibbar, RN. (2009) Study of starch granule size distribution in near isogenic lines of barley. The 16th Annual Life and Health Sciences Research Conference, University of Saskatchewan, Saskatoon, SK, Canada, March 13, 2009, pp. 66. **(Winner first place award in the Plant Sciences section)**

Personnel involved:

Investgators:

Ravindra Chibbar	10%
Monica Båga	10%
Brian Rossnagel	10%

Post-doctoral Fellows;

Omid Ansari	100%
Sarita Jaiswal	100%

Students and Technicians:

Shua Zou (Technician)	25%
Eric Asare (Graduate Student)	75%
Luke Henrichs (Summer student)	25%

Expense Statement will be sent by Financial Reporting.

